ORIGINAL ARTICLE

Specific testing for ''isolated'' anti-52 kDa SSA/Ro antibodies during standard anti-extractable nuclear antigen testing is of limited clinical value

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Aim: To ascertain whether specific testing for ''isolated'' anti-52 kDa SSA/Ro antibodies (a-SSA/Ro52) during standard anti-extractable nuclear antigen (ENA) testing is clinically useful.
Methods: 1438 consecutive sera submitted for anti-ENA testing over 1 year were evaluated for a-SSA/Ro52 using various assays.
Results: 7 of 1438 (0.48%) patients were found to have a-SSA/Ro52 without SSA/Ro60 antibodies. Subsequent testing detected a further five patients. Clinical follow-up was possible in 10/12 patients. 2 of these 10 patients had evidence of primary Sjögren's syndrome (SS) and one had systemic lupus erythematosus (SLE), with sicca symptoms and abnormal Schirmer's tests. Five other patients had sicca symptoms, of which four had abnormal Schirmer's tests.
Conclusions: ''Isolated'' anti-52 kDa SSA/Ro antibodies were detected in approximately 0.5% of standard anti-ENA requests, in which their presence was generally not associated with underlying SS or SLE. In view of the increased testing complexity and costs in detecting and confirming these antibodies, specific testing for isolated a-SSA Ro52 antibodies during standard anti-ENA testing seems to be of limited clinical value in a non-obstetric population.

The clinical associations of antibodies to the 60 kDa SSA/Ro protein are well documented and include Sjögren's syndrome (SS), systemic lupus erythematosus (SLE) and fetal–maternal autoimmune syndromes.[1] Although the 60 kDa form of SSA/Ro has been extensively studied, less is known about the 52 kDa form, and the clinical significance of autoantibodies directed against it.[1 2] Antibodies to 52 kDa SSA/Ro (a-SSA/Ro52) can exist without the presence of concomitant 60 kDa SSA/Ro antibodies (''isolated'' a-SSA/Ro52). It has been reported that isolated a-SSA/Ro52 may be the only serological marker in a subset of patients with SS and may influence patient outcomes.[3–5] There is conflicting evidence regarding the role of a-SSA/Ro52 in congenital heart block.[4 5]

There are several laboratory methods for detecting antibodies to a-SSA/Ro52, including indirect immunofluorescence (IIF), counter-current immunoelectrophoresis (CIEP), ELISA, line immunoassay (LIA) and western blot.[1 2] These differ widely in their sensitivity and specificity.[2 6] The choice of method for detecting antibodies against extractable nuclear antigens (anti-ENAs) will thus determine the detection of a-SSA/Ro52 antibodies during standard testing. In particular, gel-based immunoprecipitation methods such as double immunodiffusion or CIEP are insensitive for a-SSA/Ro52.[2 3] In a recent specimen from the external quality assurance programme of the Royal College of Pathologists of Australasia (RCPA QAP Immunology Program), the majority (~63%) of laboratories were unable to detect isolated a-SSA/Ro52 (Specimen EN7-04, June 2005). The only methods that consistently reported SSA were Orgentec (Orgentec Diagnostika GmbH, Mainz, Germany) ELISA, Inno-Lia line immunoassay (Innogenetics NV, Ghent, Belgium), Binding Site ELISA (Birmingham, UK) and Biomedical Diagnostics FIDIS (Beauburg, France). Half of the laboratories using CIEP reported an unidentified precipitin line that could not be characterised, and one laboratory reported a negative anti-ENA result.

The Division of Immunology, Queensland Health Pathology Services, receives specimens from all public hospitals throughout the state of Queensland, Australia, and has two laboratories at the Princess Alexandra Hospital (PAH) and Royal Brisbane and Women's Hospitals (RBWH). At PAH a commercial ELISA (ENAscreen, and ENAcombi Orgentec Diagnostika GmbH) is used to detect anti-ENA, whereas at RBWH in-house CIEP is used. Based on our knowledge of the ability of the above methods to detect a-SSA/Ro52, it is expected that, while the PAH laboratory would detect a-SSA/Ro52 on standard anti-ENA testing, the RBWH laboratory would not. As part of a state-wide rationalisation process, anti-ENA testing will be consolidated at the RBWH laboratory, yielding samples containing isolated a-SSA/Ro52 being considered negative by CIEP. To retain the ability to detect such sera, it would be necessary to test all sera either by Orgentec ELISA or by Inno-LIA, resulting in increased labour and higher costs. We were therefore interested in knowing whether retaining the ability to detect isolated a-SSA/Ro52 as part of our standard anti-ENA testing strategy would be of significant clinical value.

MATERIALS AND METHODS

Serum samples

A total of 1438 consecutive specimens that had been submitted for standard anti-ENA testing to the PAH laboratory over the course of 1 year (June 2000–June 2001) were analysed using a strategy that would detect anti-52 kDa SSA/Ro antibodies. This

Abbreviations: ANA, antinuclear antibody; a-SSA/Ro52, anti-52 kDa SSA/Ro antibodies; CIEP, counter-current immunoelectrophoresis; ENA, extractable nuclear antigen; IIF, indirect immunofluorescence; LIA, line immunoassay; PAH, Princess Alexandra Hospital; RBWH, Royal Brisbane and Women's Hospitals; RCPA QCP, quality assurance programme of the Royal College of Pathologists of Australasia; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome

Table 1 Results of specific anti-extractable nuclear antigen and antinuclear antibody testing

Case number	ENAcombi ELISA*	a-SSA-52 ELISA value (U/ml) †	Inno-LIA	CIEP	ANA endpoint titre and pattern‡	Clinical details
1§	SSA	>100	SSA/Ro52	Upl	1:160 Homogeneous	Primary SS
2	SSA	ND	SSA/Ro52	Upl	1:2560 Homogeneous 1:40 Speckled	Primary SS. Type I cryoglobulinaemia
3§	SSA	>100	SSA/Ro52	Neg	1:640 Homogeneous	SLE
4§	SSA Jo-1	>100	SSA/Ro52 Jo-1	Jo-1	1:40 Speckled	Polymyositis
5§	SSA	>100	SSA/Ro52 CENP-B	SSA	>1:2560 Centromere	Hepatitis C infection with some features of CREST syndrome
6	SSA	ND	SSA/Ro52	Neg	Neg	Hepatitis C infection and type III cryoglobulinaemia
7§	SSA	91	SSA/Ro52 CENP-B	Neg	1:2560 Centromere 1:2560 Nuclear membrane	Post-liver transplant for primary biliary cirrhosis
8§	SSA	>100	SSA/Ro52	Upl	1:640 Speckled	Rheumatoid arthritis with vasculitis (rheumatoid factor-positive)
9§	SSA	>100	SSA/Ro52	Upl	Neg	Rheumatoid arthritis (rheumatoid factor-negative)
10	SSA	>100	SSA/Ro52	Neg	Neg	Sensorimotor neuropathy
11	SSA	ND	SSA/Ro52	Neg	1:2560 Nucleolar	Diffuse scleroderma
12	SSA	ND	SSA/Ro52	Neg	1:40 Speckled	Chronic active hepatitis

ANA, antinuclear antibody; CENP, centromere protein B; CIEP, counter-current immunoelectrophoresis; CREST, calcinosis, Raynaud phenomenon, oesophageal dysmotility, sclerodactyly, and telangiectasia; ENA, extractable nuclear antigen; LIA, line immunoassay; ND, not done; Neg, negative; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; Upl, unidentified precipitin line.
*All specimens were positive on Orgentec ENAscreen ELISA. This ELISA does not contain CENP-B antigen.
†Cut-off value for Orgentec anti-SSA-52 ELISA = 10 U/ml.
‡Specimens were tested at an initial dilution of 1:40 using Hep-2000 slides.
§Identified from 1438 consecutive specimens submitted for standard anti-ENA testing over 1 year.

was performed by the Orgentec ENAscreen ELISA, followed by further testing with in-house CIEP, Inno-LIA, Orgentec ENAcombi ELISA and a specific Orgentec 52 kDa SSA/Ro ELISA (anti-SSA-52) as follows. All specimens were also tested for the presence of antinuclear antibodies (ANAs) by IIF on HEp-2000 slides (Immuno Concepts, Sacramento, California, USA).

In-house CIEP

Antigen extracts for CIEP

Antigen extracts were prepared from lyophilised powder and stored at −70°C before use. Calf thymus extract (in-house preparation)[7] was used to detect anti-Ro60 (SSA), anti-Sm, anti-Jo-1 and anti-ribonucleoprotein (RNP), and rabbit thymus extract (Pel-Feez Biologicals, Rogers, Arkansas, USA) was used to detect anti-LA(SSB), anti-RNP and anti-Scl70.

Antibody controls

In-house control sera containing antibodies to Ro60/SSA, LA/SSB, Sm, Jo-1, RNP and Scl70 (established against controls from the Centers for Disease Control) were used to determine lines of identity on the gels.

Gel electrophoresis

CIEP was performed by the method described by Collins *et al*.[7] In summary, 0.6% agarose in 0.25 M Barbital buffer gels were prepared, loaded with sera and antigen extract, and placed in an electrophoresis tank containing Barbital buffer (pH 8.6). Electrophoresis was performed at 10 mA per gel for 45 min. Gels were then washed in phosphate-buffered saline, and read under oblique light.

Orgentec ENAscreen, ENAcombi and a-SSA-52 ELISA

The commercial Orgentec ENAscreen, ENAcombi and a-SSA-52 ELISAs were carried out according to the manufacturers' instructions. Absorbance was read at 450 nm on a Diagnostics Pastuer ELISA plate reader. ENA positivity in the ENAscreen and ENAcombi was determined in comparison to a cut-off control and reported in a semiquantitative fashion. For the a-SSA-52 ELISA, the concentrations of a-SSA/Ro52 were calculated as arbitrary units/ml using a multipoint calibration curve, and interpreted in accordance with the manufacturers' cut-offs (normal <10 U/ml, elevated >10 U/ml).

Inno-line immunoassay

The Inno-LIA was performed and interpreted according to the manufacturer's instructions. An antibody was considered to be present if the patient serum reacted more strongly with the antigen band than the control serum provided with the kit.

Clinical assessment

This study was approved by the PAH ethics committee, and all patients gave informed consent to be clinically assessed. Permission from the treating doctor was obtained to contact the patients and enrol them in the study.

Patients with isolated a-SSA/Ro52 were contacted by telephone in April–May 2002 and asked to attend the immunology clinic. Two patients were deceased at this time, but the remaining 10 were able to attend the evaluation. Patients were evaluated for the presence of primary SS using the 2002 international criteria.[8] Schirmer's test was carried out in unanaesthetised eyes using standardised strips of filter paper (Clement Clarke, Essex, UK), with the eyes closed for a period of 5 min. The length of wetting of the strips after 5 min was recorded in millimetres, out of a maximum of 15 mm. Examination of minor salivary gland biopsy specimens and salivary flow was not performed. The medical records of the patients were used to obtain information about the presence of other conditions—for example, SLE, scleroderma, hepatitis C.

RESULTS

Laboratory findings

From the 1438 sera submitted for standard anti-ENA testing, seven specimens were found to contain a-SSA/Ro52 without anti-SSA/Ro60 (isolated a-SSA/Ro52; table 1). A further five sera were identified in subsequent testing (by identifying specimens that were positive by Orgentec Enascreen but negative on CIEP, and through follow-up testing by Inno-LIA) and included in the clinical analysis. Unfortunately, the number of sera required for screening to identify these five

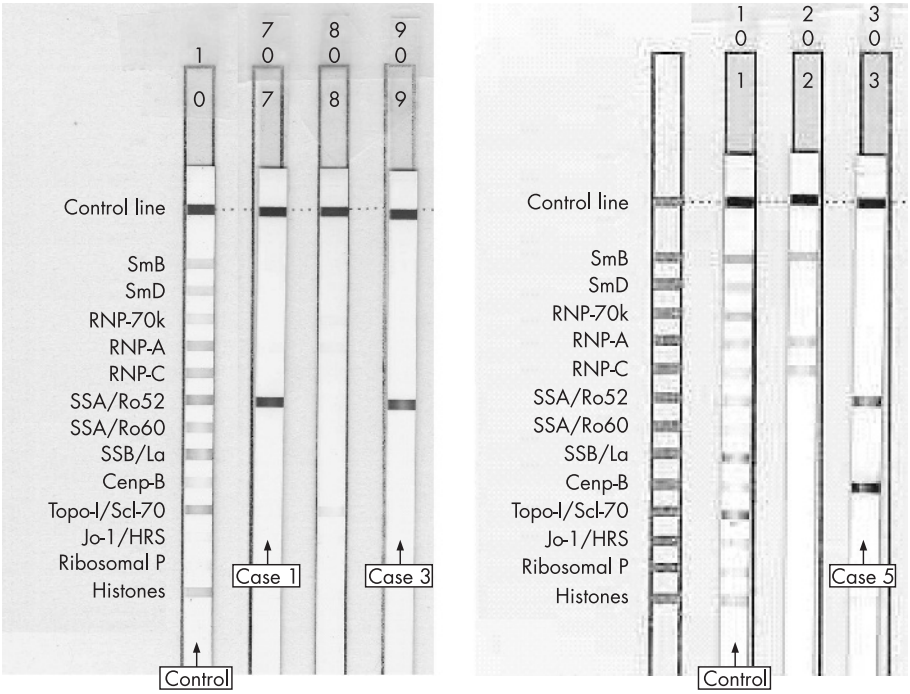

Figure 1 Sample line immunoassay results for cases 1, 3 and 5.

extras could not be determined. Statistical analysis was based on the initial seven patients. All 12 sera produced clearly positive a-SSA/Ro52 bands on Inno-LIA (fig 1). Eight of the 12 specimens were tested by the specific SSA-52 ELISA, with strong positive results. A precipitin line that identified with an anti-SSA control in the CIEP assay was detected in one of these 12 patients (case 5).

In three patients, other anti-ENA specificities were confirmed: anti-Jo-1 in case 4 (by in-house CIEP, Inno-LIA and Orgentec ENAcombi ELISA); and anti-centromere protein B in cases 5 and 7 (by Inno-LIA). In six patients (cases 3, 6, 7, 10, 11 and 12) no precipitin line was detected, and in a further four patients (1, 2, 8 and 9) a single unidentified precipitin line was detected.

ANA testing by IIF did not detect any nuclear or cytoplasmic staining at a screening dilution of 1:40 in three cases (6, 9 and 10).

Clinical findings

Of the 12 patients identified by laboratory evaluation, two were deceased before clinical assessment. Table 2 summarises the clinical features of the other 10 patients. Most cases continue to be followed up at the Immunology Outpatients Clinic, PAH, to see whether they develop features of SS or SLE.

Table 2 Clinical features, presence of sicca symptoms and results of Schirmer's test

	Case number and patient's clinical details	Presence of sicca symptoms	Schirmer's test score*
1.	Primary SS	Oral/ocular	0/15 bilaterally
2.	Primary SS. Type I cryoglobulinaemia	Oral/ocular	0/15 bilaterally
3.	SLE	Oral/ocular	0/15 bilaterally
4.	Polymyositis	Oral†	Deceased
5.	Hepatitis C infection with some features of CREST syndrome	Oral/ocular	9/15 bilaterally
6.	Hepatitis C infection and type III cryoglobulinaemia	None	>15 bilaterally
7.	Post-liver transplant for primary biliary cirrhosis	None	>15 bilaterally
8.	Rheumatoid arthritis with vasculitis (rheumatoid factor-positive)	Oral/ocular	3/15 bilaterally
9.	Rheumatoid arthritis (rheumatoid factor-negative)	None	>15 bilaterally
10.	Sensorimotor neuropathy	None†	Deceased
11.	Diffuse scleroderma	Oral/ocular	0/15 bilaterally
12.	Chronic active hepatitis	None	>15 bilaterally

SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.
*Schirmer's test score = the length of wetting of the strips after 5 min, recorded in millimetres, out of a maximum score of 15 mm.
†As the patient was deceased, the presence or absence of sicca symptoms was determined from the patient's medical records.

Of the 12 patients, 2 (cases 8 and 9) had rheumatoid arthritis, 2 (cases 1 and 2) had primary SS, 2 (cases 5 and 6) had chronic hepatitis C, 2 (cases 7 and 12) had autoimmune liver disease, 1 (case 3) had SLE, 1 (case 11) had diffuse scleroderma, 1 (case 4) had polymyositis with interstitial lung disease (deceased) and 1 (case 10) had idiopathic sensorimotor neuropathy (deceased). The clinical details of the two patients who were deceased were obtained from patient notes.

All of the three patients with SS or SLE had symptoms of the sicca syndrome and had abnormal Schirmer's tests. Four (cases 4, 5, 8 and 11) of the remaining nine patients had sicca symptoms, of which all three (cases 5, 8 and 11) who were evaluated had abnormal Schirmer's tests.

DISCUSSION

A possible limitation of our study was the methodology used to detect the presence of a-SSA/Ro52—in particular, the use of the Inno-LIA line immunoassay rather than immunoblotting using a HeLa-S100 extract, which has been historically considered as the gold-standard method for the detection of a-SSA/Ro52.[2] However, Peene *et al*[2] demonstrated that the Inno-LIA assay has an equivalent sensitivity for a-SSA/Ro52 to HeLa-S100 immunoblot. Lopez-Longo *et al*[9] also confirmed the diagnostic value of the Inno-LIA assay for the detection of a-SSA/Ro52.

In our study, there was no reliable ANA pattern associated with the presence of isolated a-SSA/Ro52. Transfection with 60 kDa Ro/SSA does not allow increased detection of isolated a-SSA/Ro52 in Hep2000 cells compared with Hep2 cells, as the former is not transfected with the 52 kDa Ro/SSA gene.[10] Indeed, ANA testing using HEp-2000 slides did not detect nuclear or cytoplasmic staining in three sera positive for isolated a-SSA/Ro52. Thus, the ANA is not a useful screening test for isolated a-SSA/Ro52.

The prevalence of isolated a-SSA/Ro52 was approximately 0.5% (7/1438) in samples submitted for standard anti-ENA testing over the initial 1-year period. Although repeat requests from patients found to have isolated a-SSA/Ro52 were excluded from the analysis, it was not possible to exclude all repeat anti-ENA requests from the analysis. Thus, the prevalence figure is likely to be even higher. In the initial 1-year period, the overall rate of ENA positivity in the 1438 samples was approximately 5%, and thus positive isolated a-SSA/Ro52 results represent approximately 10% of all positive ENA results. Other studies have suggested a much higher overall rate of ENA positivity in similar patient populations, such as 16% in the study by Peene *et al*,[2] although ENA testing was performed only on ANA-positive patients in this report. In our laboratory, ENA testing is carried out when specifically requested by the clinician. With these considerations, our results suggest that a-SSA/Ro52 would be one of the most common anti-ENA, along with anti-60 kDa SSA/Ro and anti-RNP.[2]

Despite this high frequency of isolated a-SSA/Ro52 in our population, we could not find a significant clinical benefit from detecting these antibodies. In no case did the finding of isolated a-SSA/Ro52 provide the sole explanation for symptoms, as there was adequate explanation for the symptoms in the other conditions of the patients. It is quite possible that the presence of isolated a-SSA/Ro52 modified the degree of sicca symptoms, although this study was not designed to show this. The mooted significance of a-SSA/Ro52 in congenital complete heart block was also not addressed in our study, as no patients with isolated a-SSA/Ro52 were women of child-bearing age, most likely because the PAH laboratory does not receive specimens from the major obstetric hospitals in Brisbane.

In two cases, a-SSA/Ro52 was present with hepatitis C infection. Although the international consensus statement[8] for SS excludes hepatitis C carriers, these two patients would still not have been classified as having SS, owing to the lack of clinical testing abnormalities. In one patient (case 4), co-occurrence of a-SSA/Ro52 and anti-Jo-1 was observed. This has been well described, although the clinical significance is uncertain.[6 11]

The results of the aforementioned specimen in the RCPA QAP Immunology Program (Specimen EN7-04, June 2005) question the validity of reporting specimens with isolated a-SSA/Ro52 as anti-Ro/SSA-positive (the stated target result for this specimen), given their weaker association with SS and SLE (see above). Nevertheless, in the accompanying commentary, the RCPA QAP Immunology Program mentions the introduction of a new and separate code (Ro52) for laboratories to report the presence of these antibodies.

We therefore conclude that the specific testing for isolated a-SSA/Ro52 in standard anti-ENA testing strategies is of limited clinical value in a non-obstetric population, given the additional tests and associated expense required ($A35–70 per Inno-LIA (€21.237–42.480, £14.514–29.028), more for a-SSA/Ro52 ELISA and immunoblotting).

Take-home message

Specific testing for the presence of isolated anti-SSA/Ro52 antibodies during standard anti-extractable nuclear antigen testing is of limited clinical value in a non-obstetric population.

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